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Outbreaks of *Pneumocystis pneumonia* in 2 renal transplant centers linked to a single strain of *Pneumocystis*: implications for transmission and virulence

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Abstract: **BACKGROUND:** There have been numerous reports of clustered outbreaks of *Pneumocystis pneumonia* (PCP) at renal transplant centers over the past 2 decades. It has been unclear whether these outbreaks were linked epidemiologically to 1 or several unique strains, which could have implications for transmission patterns or strain virulence. **METHODS:** Restriction fragment length polymorphism (RFLP) analysis was used to compare *Pneumocystis* isolates from 3 outbreaks of PCP in renal transplant patients in Germany, Switzerland, and Japan, as well as nontransplant isolates from both human immunodeficiency virus (HIV)-infected and uninfected patients. **RESULTS:** Based on RFLP analysis, a single *Pneumocystis* strain caused pneumonia in transplant patients in Switzerland (7 patients) and Germany (14 patients). This strain was different from the strain that caused an outbreak in transplant patients in Japan, as well as strains causing sporadic cases of PCP in nontransplant patients with or without HIV infection. **CONCLUSIONS:** Two geographically distinct clusters of PCP in Europe were due to a single strain of *Pneumocystis*. This suggests either enhanced virulence of this strain in transplant patients or a common, but unidentified, source of transmission. Outbreaks of PCP can be better understood by enhanced knowledge of transmission patterns and strain variation.

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Outbreaks of *Pneumocystis* Pneumonia in 2 Renal Transplant Centers Linked to a Single Strain of *Pneumocystis*: Implications for Transmission and Virulence

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(See the Editorial Commentary by de Boer, on pages 1445–7.)

Background. There have been numerous reports of clustered outbreaks of *Pneumocystis* pneumonia (PCP) at renal transplant centers over the past 2 decades. It has been unclear whether these outbreaks were linked epidemiologically to 1 or several unique strains, which could have implications for transmission patterns or strain virulence.

Methods. Restriction fragment length polymorphism (RFLP) analysis was used to compare *Pneumocystis* isolates from 3 outbreaks of PCP in renal transplant patients in Germany, Switzerland, and Japan, as well as nontransplant isolates from both human immunodeficiency virus (HIV)–infected and uninfected patients.

Results. Based on RFLP analysis, a single *Pneumocystis* strain caused pneumonia in transplant patients in Switzerland (7 patients) and Germany (14 patients). This strain was different from the strain that caused an outbreak in transplant patients in Japan, as well as strains causing sporadic cases of PCP in nontransplant patients with or without HIV infection.

Conclusions. Two geographically distinct clusters of PCP in Europe were due to a single strain of *Pneumocystis*. This suggests either enhanced virulence of this strain in transplant patients or a common, but unidentified, source of transmission. Outbreaks of PCP can be better understood by enhanced knowledge of transmission patterns and strain variation.

Pneumocystis jirovecii continues to be an important, often fatal, cause of *Pneumocystis* pneumonia (PCP) in a wide spectrum of immunosuppressed patients including patients with human immunodeficiency virus (HIV) infection and patients who have received human stem cell or solid organ transplants [1, 2]. Although prophylaxis has been very effective in preventing PCP

in HIV infection, identification of patients who are at risk for PCP and thus suitable candidates for prophylaxis in non-HIV populations can be more difficult. Notable outbreaks of PCP have occurred, especially in renal transplant patients over the past 2 decades, primarily from centers in Europe and Japan [3–9]. Renal transplant patients in the recent era may well have been susceptible to PCP because of inconsistent use of anti-*Pneumocystis* prophylaxis at many centers in the context of changing immunosuppressive regimens. However, the dramatic occurrence of clusters that are geographically and temporally distinct suggests that special circumstances may exist where renal transplant patients are uniquely susceptible to infection, possibly due to epidemiologic factors, such as dedicated clinics

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for transplant patients, or to a unique, potentially more virulent strain of *Pneumocystis*.

We have recently developed a typing technique using restriction fragment length polymorphism (RFLP) analysis that has allowed us to demonstrate substantial diversity among *Pneumocystis* isolates, both in HIV-infected and uninfected patients [10]. A remarkable feature of our studies is the tremendous variability seen in the RFLP patterns: no 2 patients with sporadic cases of PCP showed the same pattern, suggesting that each case was caused by a unique strain of *Pneumocystis*. However, in contrast to this experience with sporadic cases, using this technique we were able to confirm that an outbreak of PCP in Germany in 2006 was caused by a single *Pneumocystis* strain [7, 10]. These studies support the high discriminatory power of this typing technique. The availability of samples from additional outbreaks in renal transplant centers in Zurich, Switzerland (2006–2007) [5], and Nagoya, Japan (2004–2008) [8], provided an opportunity to study strain differences among patients and centers and to compare strains causing disease within Europe with those outside of Europe.

MATERIALS AND METHODS

Patients

The epidemiology, patient characteristics, and molecular analysis of *P. jirovecii* isolates using single-nucleotide polymorphism (SNP) or multilocus sequence typing (MLST) analysis for the outbreaks of PCP in Munich, Zurich, and Nagoya and RFLP analysis for the Munich outbreak have been previously reported [5, 7, 8, 10]. Extracted DNA that included samples from patients who were identified as being part of the outbreak as well as local nonoutbreak (control) PCP samples were provided to the National Institutes of Health (NIH) as coded samples. RFLP analysis was performed in a blinded manner, and the code from each center was not broken until the analysis from that center was complete. Samples from all 11 patients from Zurich (7 outbreak and 4 control) and all 10 from Nagoya (9 outbreak and 1 control) that had previously undergone molecular typing analysis were made available for our studies. To allow confirmation of the results for the latter, a second, recoded aliquot of the same 10 samples was provided and again analyzed in a blinded manner. Our previous analysis of samples from Munich included 13 of the 16 outbreak patients who had undergone molecular typing analysis as well as 6 control samples [10]. The guidelines of the US Department of Health and Human Services and the NIH were followed in the conduct of these studies.

Polymerase Chain Reaction Amplification and RFLP Analysis

As a first step, the *msg* gene copy number for each DNA sample was quantified by a previously described real-time quantitative

polymerase chain reaction (qPCR) assay [11]. In previous studies we have shown that for reproducibility, a minimum of approximately 1000 *msg* copies needs to be used per RFLP PCR reaction [10]. Subsequently, *msg* variable region (~1.3 kb) was amplified by a seminested PCR as previously described [10], using primers GK 472, GK 452, and GK 195. A minimum of 1000 *msg* copies per reaction was used whenever possible. The PCR was performed using HotStart Taq DNA polymerase (Qiagen), and the conditions were 15 minutes at 95°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 4 minutes (for the first round) or 2 minutes (for the second round) at 72°C, with a final extension of 10 minutes at 72°C.

RFLP analysis was performed as previously described [10]. Agarose gel electrophoresis was used to verify that amplification was successful. PCR products were purified using the QuickStep 2 PCR Purification Kit (Edge BioSystems, Gaithersburg, Maryland), digested with *Dra*I and *Hpy*188I restriction enzymes for 6 hours at 37°C, and analyzed on a 1% or 2% tris-borate-ethylenediaminetetraacetic acid agarose gel following staining with SYBR green (Molecular Probes, Eugene, Oregon), as well as by Southern blotting. For the latter, the blot was hybridized with a digoxigenin-labeled DNA probe (PCR DIG Probe Synthesis Kit; Roche, Indianapolis, Indiana) of ~1.3 kb that was an equal mixture of PCR products from 4 *P. jirovecii* isolates; hybridization signal was detected using alkaline phosphatase-conjugated anti-digoxigenin antibody and CDP-Star (Roche) and a Kodak Image Station 440CF (PerkinElmer, Waltham, Massachusetts). Each run included Lambda/HindIII molecular weight markers or a single clinical sample (sample number 385) as an internal control.

The gels were analyzed using BioNumerics software version 4.01 (Applied Maths, Austin, Texas) as previously described [10]. The pattern of banding among different gels/blots was normalized using Lambda/HindIII molecular weight markers. The Dice coefficient was used to analyze the similarity of the patterns of bands with a position tolerance of 1.9% [12]. The unweighted pair group method with average linkages was used by the BioNumerics software for cluster analysis. DNA samples with banding patterns with 100% similarity (Dice coefficient = 1) were considered to be identical. Standard deviations of the branches in the cluster were obtained using the BioNumerics “Clustering/Calculate error flags” setting and represent the reliability and internal consistency of the branch.

26S Ribosomal RNA and Tandem Repeat Analysis

Amplification and sequencing of the 26S ribosomal RNA (rRNA) gene and tandem repeats in the intron of the *msg* expression site were performed as previously described [5, 7, 13, 14].

RESULTS

Analysis of the Outbreak in Zurich

Our initial goal was to determine whether RFLP analysis could demonstrate that a single strain of *Pneumocystis* was responsible for the outbreak of PCP in Zurich. Two *P. jirovecii* DNA samples from a single patient had a very low *msg* copy number and could not be amplified for RFLP analysis. Of the remaining 10 samples (10 patients) analyzed in a blinded manner, 7 had an identical pattern by RFLP analysis when digested with either DraI or Hpy188I restriction enzymes and evaluated by either agarose gel electrophoresis or Southern blotting (Figure 1). After breaking the code, these 7 patients were confirmed to be part of the renal transplant outbreak. The remaining 3 samples had a different pattern with each enzyme and were confirmed to be from control, nonoutbreak patients.

Given that the outbreaks in both Munich and Zurich were in renal transplant patients, we sought to determine whether the same *P. jirovecii* strain was responsible for these outbreaks. Because all 14 previously studied German samples gave an identical RFLP pattern [7], we included a single representative German isolate in each gel for the RFLP analysis of the Swiss isolates. As can be seen in Figure 1, the RFLP pattern for the German isolate (lane G) was identical to that of the Swiss outbreak isolates with both restriction enzymes. Thus, the same *P. jirovecii* strain was apparently responsible for 2 separate and geographically distinct outbreaks in renal transplant patients.

In the original reports of the 2 outbreaks, MLST was performed using the same set of 4 gene targets [5, 7]. For 3 of the 4 genes, the same allele was identified in transplant patient isolates in both centers: alleles B, 7, and 1 for ITS1, mt26S, and β -tubulin, respectively. For the fourth gene, 26S rRNA, each center reported identification of a new allele, designated as allele 4 [7] and allele 5 [5]. To determine if these alleles were identical, we sequenced 1–2 isolates from each outbreak. We found that both isolates had an identical sequence that differed from the reference, allele 1, at positions 301–306: allele 1 had TACTCT in these positions, while the outbreak isolates had ACTCTT. Thus, MLST analysis provided further evidence that the 2 outbreaks were caused by a single strain. Sequencing of a limited number of subcloned *msg* genes from Swiss and German isolates provided additional support that they are the same strain (data not shown).

We were not able to undertake a formal epidemiologic investigation and thus do not know if there was any link between either patients or healthcare providers at the 2 centers.

Analysis of the Outbreak in Nagoya

Given that 2 outbreaks in renal transplant patients in Europe were caused by a single *P. jirovecii* strain, we wanted to determine

whether renal transplant patients were uniquely susceptible to this strain by examining isolates from a third outbreak that occurred in Nagoya, Japan. We obtained 10 DNA samples from this outbreak [8], but only 4 could be amplified for RFLP analysis; the remaining 6 samples had very low (<20) *msg* copies/ μ L. In each experiment we included representative samples from Switzerland (S) and Germany (G) to compare the RFLP pattern from different outbreaks.

Three of the 4 amplifiable DNA samples from Japan that were analyzed in a blinded manner showed an identical RFLP banding pattern when they were digested with DraI or Hpy188I (Figure 2). One sample (38 *msg* copies/ μ L, ~ 1000 *msg* copies per assay) showed a different RFLP pattern both with DraI and Hpy188I when compared with the other samples. None of the 4 samples showed an RFLP pattern that was identical to the Swiss or German pattern (Figure 2). After breaking the code, all 4 samples were found to be from renal transplant patients. To verify these results, a second aliquot of all 10 samples (recoded) was sent for RFLP analysis, again in a blinded manner. Only 3 samples could be amplified for RFLP analysis; all 3 showed an identical pattern to each other and to the 3 identical samples from the first round. Thus, the same strain of *P. jirovecii* appears to be responsible for 3 of these infections in renal transplant patients, but this strain is different from the strain that caused the 2 European outbreaks.

Figure 3 shows a dendrogram of samples from the current study (representative outbreak as well as control samples) together with samples from endemic cases included in a prior publication [10]. The cases from the European and Japanese outbreaks cluster together but separately from each other as well as from the endemic cases.

To extend our observations we examined 1 representative German sample and 2 representative Swiss samples from renal transplant patients using a second typing method based on variation in the number and sequence of tandem repeats in the *msg* expression site [14]. In addition, we were able to amplify all 10 Japanese samples for this analysis, presumably because the region being amplified was shorter than that required for RFLP analysis, which allows a higher amplification efficiency. All 13 samples had 3 tandem repeats with an identical sequence. Thus, RFLP analysis provided greater discrimination than tandem repeat analysis for distinguishing among the strains. However, although 9 Japanese samples were identical throughout the sequenced region (~ 250 bp), the 10th sample, which was from the nontransplant patient (and which could not be amplified for RFLP analysis), had 2 SNPs outside the tandem repeat region that differed from the other samples (Figure 4). This is consistent with disease resulting from infection with a strain different from the primary outbreak strain in Japan.

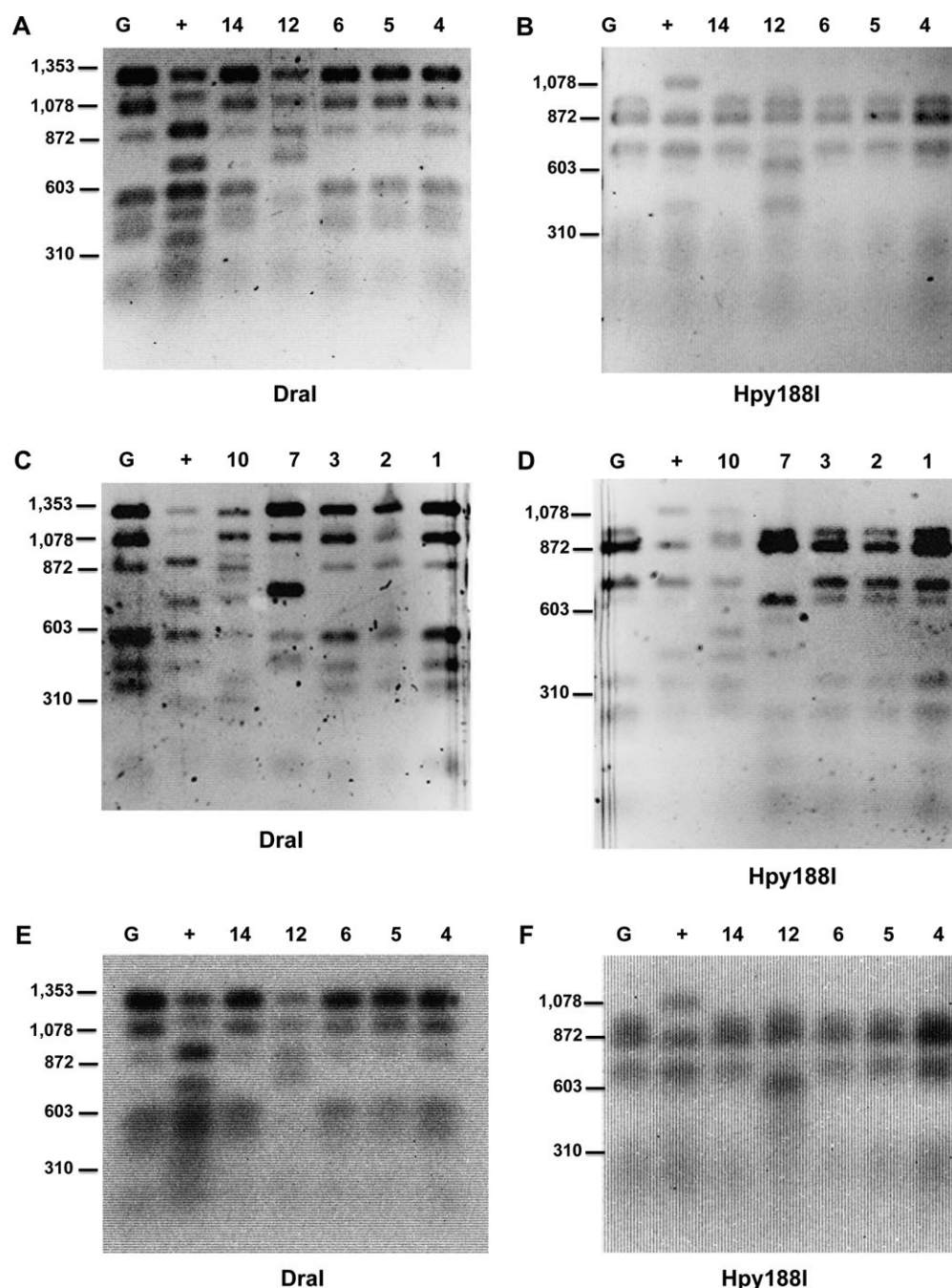


Figure 1. Restriction fragment length polymorphism (RFLP) analysis of *Pneumocystis* samples from Zurich, Switzerland. A–D, The RFLP pattern following agarose gel electrophoresis for the 10 samples that could be amplified for analysis. Labels at the top represent the individual samples. A and C, Gels were run following digestion with Dral. B and D, Gels were run following digestion with Hpy188I. Samples 1–6 and 14 are from renal transplant patients, and samples 7, 10, and 12 are from control patients. The letter G denotes a representative sample from the outbreak in Munich, Germany; + is a positive control. With both enzymes, the RFLP patterns of the renal transplant patients are identical to each other and to the German sample, whereas the control patients showed patterns that were different from each other as well as from the transplant patients. E and F, Southern blots of the gels from panels A and B, confirming the results of the gel analysis. Molecular weight markers are indicated on the left.

DISCUSSION

RFLP analysis provides an important new tool for studying the epidemiology of *Pneumocystis* infection. In general, each case of

sporadic PCP, whether in HIV-infected patients or in other immunosuppressed patients, is caused by a unique strain of *Pneumocystis* as determined based on RFLP analysis. However, in the current study, we have demonstrated that 2 geographically

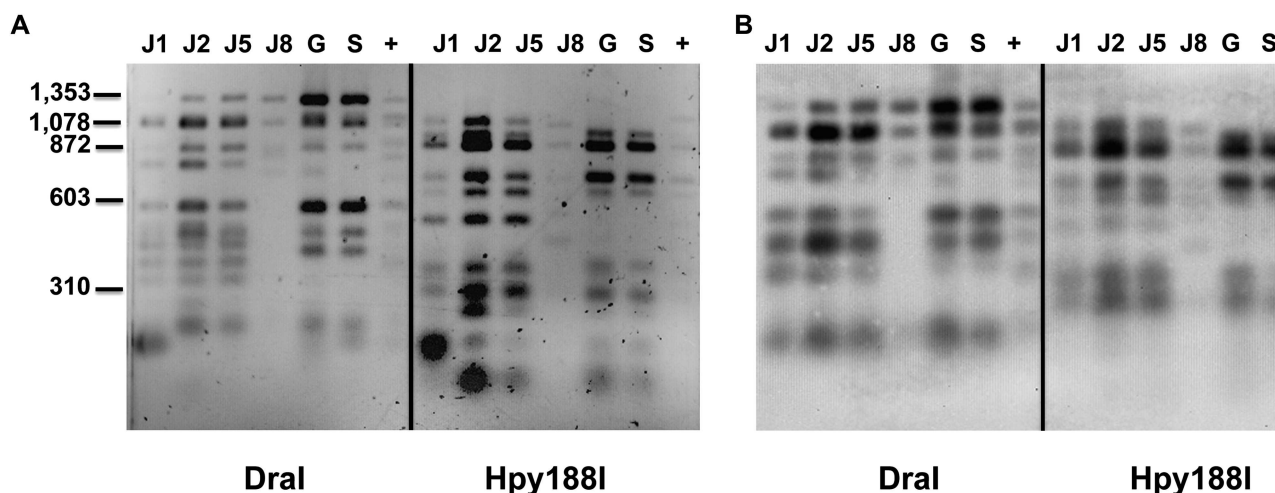


Figure 2. Restriction fragment length polymorphism (RFLP) analysis of *Pneumocystis* samples from Nagoya, Japan. The RFLP pattern following agarose gel electrophoresis (A) (following digestion with Dral on the left and Hpy188I on the right) and following Southern blotting (B), for the 4 samples that could be amplified for analysis. Labels at the top represent the individual samples. All 4 samples (J1, J2, J5, and J8) are from renal transplant patients. The letter G denotes a representative sample from the outbreak in Munich, Germany; the letter S denotes a representative sample from the outbreak in Zurich, Switzerland; + is a positive control. Samples J1, J2, and J5 showed a pattern identical to each other but different from the G and S samples, whereas sample J8 was different from all other samples. Molecular weight markers are indicated on the left.

distinct outbreaks of PCP involving renal transplant recipients were due to a single, unique *Pneumocystis* strain that we had not previously identified in other populations [10]. In line with our previous observations, the 9 contemporaneous nonoutbreak isolates (6 from Germany [10] and 3 from Switzerland) all showed unique RFLP patterns. Thus, although the number of nonoutbreak isolates studied at each site is small, the outbreaks caused by the European Renal Transplant (ERT) strain do not appear to simply represent infection with a predominant, locally circulating strain. Additional analyses of larger numbers of isolates, both from endemic and epidemic cases, as well as colonized or subclinically infected individuals, will more definitively answer this question.

MLST analysis further supports these results: we have reconciled the differences originally reported in 26S rRNA alleles by showing that isolates from both outbreaks had the same allele. Original sequencing data from the German outbreak confirmed this as well. Thus, isolates from both outbreaks have an identical allele in all 4 genes. We were unable to find any information that epidemiologically linked patients at the German center and the Swiss center, which are >300 km apart.

We explored the possibility that this *Pneumocystis* isolate might have a unique association with renal transplant recipients in general, but found that 4 cases in a renal transplant center in Japan had disease due to a different strain. Thus, the ERT strain is not the only strain to cause disease in renal transplant recipients. Two outbreaks recently reported from northwest England

also are likely not caused by the same strain, given that they have different mt26S alleles [15]. Similarly, a 2010 outbreak reported from Australia also appears to be caused by a different strain based on MLST, although RFLP analysis of these isolates would be needed to definitively confirm this [16].

Chemoprophylaxis with trimethoprim-sulfamethoxazole or an alternative agent [17] would likely have prevented a substantial fraction of these cases. However, due in part to the low incidence of PCP in the period preceding the outbreaks, none of the patients in these outbreaks received PCP prophylaxis (although a subset of patients in 1 study received short courses of trimethoprim-sulfamethoxazole for urinary tract infection prophylaxis). Following the institution of routine prophylaxis at all 3 centers, the incidence of PCP decreased markedly [5, 7, 8]. Guidelines for the management of renal transplant patients currently incorporate routine anti-PCP prophylaxis [18].

Recently developed typing methods have led to important advances in our understanding of the epidemiology of *Pneumocystis*. Many patients appear to be infected with multiple strains of *Pneumocystis* simultaneously [14, 19]. Although it was long thought that PCP represented reactivation of latent infection that had occurred much earlier in life, possibly during infancy, recent studies have suggested that many sporadic cases in HIV-infected patients result from recently acquired infection [20].

The demonstration that outbreaks of PCP at 1 or more renal transplant centers were caused by a single strain of *Pneumocystis* provides unambiguous evidence that disease can result from

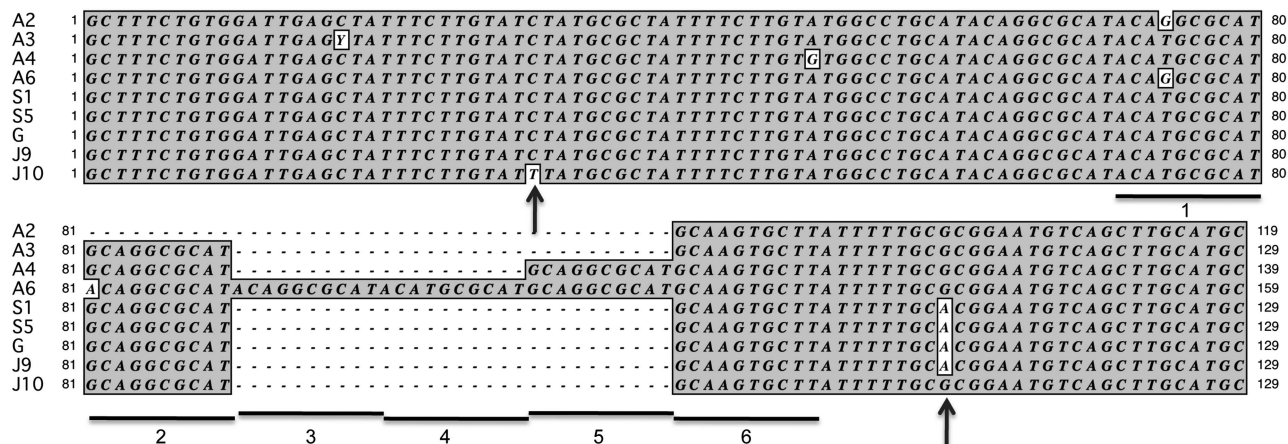


Figure 4. Sequence analysis and alignment of a region in the intron of the *msg* expression site that includes tandem repeats, which are underlined. Shown are results for 2 Swiss samples (S1, S5), a German sample (G), and 2 Japanese samples (J9, J10). Samples J1–J8 (not shown) were identical in sequence to sample J9. For comparison are 4 sequences with 2, 3, 4, or 6 tandem repeats (A2–A6) obtained from a single patient from the United States [14]. Although restriction fragment length polymorphism analysis identified differences between the Japanese and European isolates, in this region the sequences from all renal transplant patients from the 3 countries were identical. The isolate from a nontransplant Japanese patient (J10) differed from the transplant isolates at 2 positions indicated by the arrows.

recent infection. The alternative explanation, that all individuals were infected during infancy with the same *Pneumocystis* strain that subsequently reactivated during immunosuppression, appears highly unlikely given the tremendous strain diversity we have previously found by RFLP typing [10].

What is the mechanism of transmission of *Pneumocystis* in these outbreaks? Animal studies have demonstrated that transmission is via the respiratory route, and *Pneumocystis* organisms have been identified in the air near infected patients and animals [21–23]. *Pneumocystis* species have a strict host specificity, and thus human infection does not represent a zoonosis. To date, there is no convincing evidence for an environmental source of infection, although such a source cannot be ruled out definitively at present. For all 3 outbreaks included in this study, the initial reports were able to identify potential contacts between infected patients [5, 7, 8]. Thus, it seems likely that the organism was transmitted from other infected patients or alternatively that a healthcare worker or patient may have been persistently colonized or had a subclinical infection that allowed transmission to a more susceptible population. The fact that at least 21 cases in 2 centers in Europe (amplifiable DNA

was unavailable for additional outbreak cases) were due to a single strain raises the possibility that this strain is unusually virulent for the renal transplant population, although the occurrence of outbreaks caused by apparently different strains makes this less likely. The outbreaks may result from a combination of these factors, which are not mutually exclusive.

Whether respiratory isolation of infected patients would decrease the risk of transmission is unknown, because in animals the incubation time following exposure to development of severe infection may be 2–3 months [24]. Nonetheless, given the clear demonstration that infection can be transmitted among susceptible patients, potentially susceptible patients should not be exposed to patients with active PCP to minimize the risk of such transmission. Alternatively, such patients may be provided with anti-*Pneumocystis* prophylaxis. However, given the difficulty in clearly defining risk for *Pneumocystis* pneumonia in many non-HIV populations, it does not seem feasible to provide all such patients with timely prophylaxis.

The link between the 2 European outbreaks is unidentified at present. Additional studies comparing the strains responsible for outbreaks in renal transplant patients at other centers both in

Figure 3 continued. included in a prior publication [10]. Seventeen samples are from the current study and include 4 representative samples from the outbreaks and the 9 control samples from Switzerland (Sw) and Germany (Ge), as well as the 4 outbreak samples from Japan (Ja). The Dice coefficient was used to calculate similarities, and unweighted pair group method with average linkages was used for cluster analysis. The position tolerance was 1.9%. The percent similarity scale is shown above the dendrogram and indicated by the numbers at the individual nodes. SDs of the branches are indicated by the gray bars. For branches without a bar, the SD was 0. The samples from the outbreaks in Europe and Japan form unique clusters that are boxed. The control samples from Europe and the outbreak sample from Japan that had a different RFLP pattern are indicated by a +. As previously reported, 6 of the paired samples with 100% identity represent samples from the same patient collected at different times [10].

Europe and elsewhere, as well as outbreaks in other susceptible populations, are needed to better define the role that the ERT strain plays in causing disease in susceptible populations. It will be important to determine if this strain has biological properties that allow it to uniquely infect renal transplant patients and, if so, to better understand what these properties are.

Outbreaks of life-threatening disease can have a potentially devastating impact on immunosuppressed populations. These outbreaks emphasize the need to develop better parameters for determining susceptibility to PCP so that prophylaxis can be continued during periods of enhanced susceptibility. These outbreaks also emphasize the importance of expanding our knowledge of biological factors that might enhance organism virulence and transmission factors that might increase the risk that susceptible patients will develop disease.

Notes

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